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IMPACT OF TOXIC ORGANIC CHEMICALS ON THE KINETICS OF ACETOCLASTIC METHANOGENESIS

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IMPACT OF TOXIC ORGANIC CHEMICALS ON THE KINETICS OF ACETOCLASTIC METHANOGENESIS

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ABSTRACT

A knowledge of the effect of toxic organic chemicals on the biotransformation characteristics of organic co-substrates is essential for predicting the impact of these chemicals in anaerobic processes. To address this need, bench-scale tests were conducted to assess the impact of toxic organic chemicals on the kinetics of acetoclastic methanogenesis and to determine the relationship between kinetic parameters obtained under non-toxic and toxic conditions. The procedure involved adding toxicant at three concentration levels to cultures transferred from an acetate-enriched Master Culture Reactor. A control without toxicant was included for comparison and a seed blank was included to provide a basis for correcting for background gas production. Unacclimated cultures were used to minimize the biodegradation of the toxic organic chemicals throughout the duration of the test. Ten milliliter samples were removed from each bottle at frequent intervals for at least 24 hours, transferred to sealed, septum-capped vials and centrifuged to remove solids. Five milliliters of supernatant were then transferred to clean 5 mL septum-capped vials containing one drop of concentrated sulfuric acid. The supernatant samples were analyzed for acetate and scluble toxicant residual by gas chromatography.

A computer model based on a finite difference non-linear least squares algorithm was used to calculate the kinetic parameters by obtaining a best-fit of the experimental data to the Michaelis-Menten substrate utilization model. Sensitivity analysis was used to predict (1) whether the parameters could be uniquely estimated for a given set of initial conditions (i.e. initial substrate and microorganism concentrations), (2) the relative precision of the estimated parameters, and (3) the portion of the uptake curve over which the model was most sensitive to changes in the parameters. The latter was then used for designing experiments for optimum parameter estimates. This approach resulted in considerable savings of time and resources.

Resulting kinetic coefficients revealed significant changes in both the maximum rate of acetate conversion, k, and the half-velocity coefficient, K_s, when using both chlorophenols and chloroanilines. Therefore, mixed inhibition was occurring. However, non-competitive mechanisms predominated with chemicals containing the 2-chloro functional group while competitive mechanisms seemed to predominate with the 3- and 4-chloro functional groups.

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INTRODUCTION

Anaerobic microbial reactions have received increased attention over the last decade as a potential means of degrading complex organic chemicals. This has resulted in significant advances in identifying the principal microorganisms involved in the anaerobic degradation process, unravelling the complex degradative pathways used by these microorganisms, and demonstrating the potential for anaerobic degradation of a large number of compounds. Despite these advances, there is limited information regarding the impact of toxic organic chemicals on the kinetics of organic co-substrate degradation and a lack of definition of the various chemical, physical and biological factors that may influence the nature and extent of inhibitory effects. Such knowledge is essential for predicting the impact of toxic chemicals on anaerobic wastewater treatment processes and preventing inefficient treatment or potentially costly upsets of treatment plant operations.

To address the need for better definition of toxicant-substrate interactions, bench-scale tests were conducted by the authors to assess the impact of toxic organic chemicals on the intrinsic kinetics of acetoclastic methanogenesis and to determine the relationship between kinetic parameters obtained under toxic and non-toxic conditions. The overall objective was to relate the effects of toxic organic chemicals on the kinetics of acetate degradation to the chemical classification and molecular structure of the toxicants. Tests conducted to date by other researchers have utilized cultures from a variety of sources, and grown under a variety of environmental conditions, making comparison of test results very difficult. Therefore, one objective of the present study was to provide a controlled culture and uniform procedures for all tests.

BACKGROUND

Anaerobic treatment reactions involve four main stages of biodegradation each requiring unique metabolic reactions and possibly a unique physiological group of bacteria. A general scheme for the interrelationships between these steps is shown in Figure 1 (Wilkie and Colleran, 1986). In the first step, complex organic materials such as fats, proteins, and carbohydrates -- which usually exist in a solid or particulate form -- are hydrolyzed to form soluble products. These soluble products then undergo fermentation to form fatty acids, alcohols, carbon dioxide, and hydrogen. During these first two stages, organic materials are converted almost entirely to organic acid intermediates and bacterial cells so that little organic mineralization occurs. In the third stage, acetogenic bacteria further convert the fermentation products into hydrogen, carbon dioxide, and acetate.

The fourth stage involves two physiologically different groups of methane-forming bacteria: acetoclastic methanogens decarboxylate acetate to form methane and carbon dioxide while the hydrogenotrophic methanogens produce methane from the reduction of carbon dioxide with hydrogen.

In view of this complexity, the authors have adopted the approach of dividing the anaerobic biotransformation sequences into specific reactions that can be isolated and studied separately or in a stepwise manner, working from simple and known reactions toward the more complex situations as illustrated in Figure 2. Figure 2A shows the acetoclastic methanogenic reaction which represents the terminal conversion of acetate to methane and carbon dioxide, and accounts for about seventy percent of the energy flow in the degradation of complex organic chemicals. The stoichiometry of this reaction is well known as are the biomass yield and decay characteristics (Lawrence and McCarty, 1969; Montgomery, 1984).

Addition of one simple acetogenic intermediate -- ethanol -- increases the complexity of the conversion process by adding at least four other reaction pathways (Figure 2B). Ethanol is first converted to acetate and hydrogen by syntrophic acetogens. The acetate so formed generally is considered to be converted to methane and carbon dioxide by the same route as shown in Figure 2A. However, the hydrogen may be converted to methane directly by the hydrogenotrophic methanogens or to acetate by the homoacetogens depending on such factors as environmental conditions and culture mix (Wolfe, 1983; Wilkie and Colleran, 1986). Little is known about the kinetics of these hydrogen conversion reactions or the factors affecting their balance. Under severe environmental stress, such as may be caused by toxic organic chemicals and severe loading conditions, the hydrogen and acetate can be combined to form propionic and butyric acids, or both, through the action of metabolic equilibrium reactions (Young and Tabak, 1989; Smith and McCarty, 1989). While the overall stoichiometry of ethanol conversion is reasonably well known, little is known about the kinetics of interacting reactions, and the methods to identify and measure the active biomass responsible for each reaction presently are not available.

Theoretical Approach

The theory of biological growth is based on two fundamental relationships: (1) the relationship between the concentration of a growth-limiting substrate and its rate of consumption, and (2) the relationship between the net microorganism growth rate and the substrate utilization rate. These relationships generally are expressed respectively as:

$$-dS/dt = k\,S\,M/(K_s + S) \qquad (1)$$

and

$$dM/dt = [Y\,k\,S/(K_s + S) - K_d]\,M \qquad (2)$$

where:

$-dS/dt$ = rate of substrate utilization, mass/time
dM/dt = net growth rate of microorganisms, mass/time
M = active microorganism concentration, mass/volume
S = concentration of growth-limiting substrate in solution, mass/volume
k = maximum specific substrate utilization rate, $time^{-1}$
K_s = half-velocity coefficient, mass/volume
Y = growth yield coefficient, mass/mass
K_d = decay coefficient, $time^{-1}$

Numerous mathematical techniques have been used by previous investigators for estimating the kinetic parameters in Equations 1 and 2, with varying degrees of success (Knowles et al, 1965; Stratton and McCarty, 1969; Gates and Marlar, 1968; Ong, 1983; Corman and Pave, 1983; Robinson and Tiedje, 1983; Montgomery, 1984). In this study, a novel finite-difference model was developed as follows:

Model Development

Equations 1 and 2 are illustrated graphically in Figure 3 for a batch substrate uptake reaction in which an initial substrate concentration, S_o, is added to a culture containing an active biomass concentration, M_o. The substrate concentration decreases as uptake occurs in accordance with Equation 1 while biomass changes are described by Equation 2. The model was developed on the basis of the following assumptions:

1. The substrate utilization and biomass curves are linear over a very small time interval Δt.

2. The operating substrate and biomass concentrations in Equations 1 and 2 are the average concentrations over Δt_{i+1} designated $\bar{S}_{i+1}$ and $\bar{M}_{i+1}$ respectively.

The differential forms of Equations 1 and 2 can be approximated by using discrete (finite difference) terms as follows:

$$-\frac{\Delta S_{i+1}}{\Delta t_{i+1}} = \frac{k\, S_{i+1}\, M_{i+1}}{(K_s + \bar{S}_{i+1})} \tag{3}$$

and

$$\frac{\Delta M_{i+1}}{\Delta t_{i+1}} = \frac{Y(-\Delta S_{i+1})}{(\Delta t_{i+1})} - K_d \bar{M}_{1+1} \tag{4}$$

where:

$$\bar{S}_{i+1} = (S_i + S_{i+1})/2 = S_i + \Delta S_{i+1}/2 \tag{5}$$

$$\bar{M}_{i+1} = (M_i + M_{i+1})/2 = M_i + \Delta M_{i+1}/2 \tag{6}$$

Substituting Equations 5 and 6 for $\bar{S}_{i+1}$ and $\bar{M}_{i+1}$ into Equations 3 and 4 and simplifying gives the following equations which can be solved simultaneously for ΔS_{i+1} and ΔM_{i+1}:

$$-\Delta S_{i+1} = \frac{0.5\, k\, (2S_i + \Delta S_{i+1})(2M_i + \Delta M_{i+1})(\Delta t_{i+1})}{(2K_s + 2S_i + \Delta S_{i+1})} \tag{7}$$

$$\Delta M_{i+1} = \frac{-2(Y\, \Delta S_{i+1} + K_d M_i\, \Delta t_{i+1})}{(2 + K_d\, \Delta t_{i+1})} \tag{8}$$

Substituting Equation 8 in Equation 7 reduces the expression for ΔS_{i+1} to a quadratic equation of the form:

$$A\,(\Delta S_{i+1})^2 + B\,(\Delta S_{i+1}) + C = 0 \qquad (9)$$

having the solution:

$$\Delta S_{i+1} = \frac{-B + (B^2 - 4\,A\,C)^{1/2}}{2\,A} \qquad (10)$$

where: $A = 2[\Delta t_{i+1}(k\,Y - K_d) - 2]$

$B = 4[\Delta t_{i+1}\,k\,(M_i(1 + \Delta t_{i+1}\,K_d + S_i\,Y)) - (K_s + S_i)(2 + K_d\,\Delta t_{i+1})]$

$C = -8\,\Delta t_{i+1}\,k\,S_i\,M_i$

Starting with the initial substrate concentration S_o, and the initial active biomass concentration M_o, the program calculates ΔS_{i+1} and ΔM_{i+1} for specified values of Δt_{i+1}, k, K_s, Y and K_d. Sequential values of S_{i+1} and M_{i+1} can then be calculated as follows:

$$S_{i+1} = S_i + \Delta S_{i+1} \qquad (11)$$

and

$$M_{i+1} = M_i + \Delta M_{i+1} \qquad (12)$$

Hence the progress of the substrate utilization and biomass curves can be completely described for i = 0,1,.....n.

The model uses a finite-difference non-linear least squares algorithm to obtain the kinetic parameters that provide a best-fit of Equations 7 and 8 to experimentally measured data. To minimize the errors associated with multi-parameter estimation from a single curve (Wang, 1988), only two parameters, k and K_s, were considered as unknowns. Values of Y and K_d for acetate-enriched cultures were obtained from published data obtained under conditions similar to those of the author's study (Montgomery, 1984; Lawrence and McCarty, 1969). The biomass concentration was determined from volatile suspended solids (VSS) measurements and the initial active fraction, M_o, was found to be about 91 percent of the total volatile suspended solids using methods described by Young (1982). The model requires initial estimates for k and K_s, which are then changed, using a two-dimensional grid search, until the residual sum of squares of the differences between the experimentally measured and predicted substrate concentrations are minimized.

Kinetic parameters, k and K_s, were first determined for a control culture under non-toxic conditions to serve as a "baseline". The effect of toxic organic compounds on the baseline kinetic parameters was determined from cultures that were spiked with toxic organic compounds while maintaining the same environmental conditions as used in the control. A new set of kinetic parameters was obtained for each toxicant level. This procedure was repeated for six compounds representing two homologous series of chloro-substituted phenols and chloro-substituted anilines.

Good experimental design together with appropriate mathematical techniques for analyzing the data are extremely crucial for accurately estimating kinetic parameters from batch culture data. One of the factors that must be taken into

consideration is the proper choice of the ratio of initial substrate concentration to active microorganism concentration to cause the reactions initially to be zero order. This is the region in which k is most sensitive to the accuracy and volume of data collected (see Figure 3). Also a sufficient number of points must be collected at substrate concentrations near K_s for accurate determination of K_s (Montgomery, 1984). This is the region in which K_s is most sensitive to the accuracy and volume of data collected (see Figure 3). Also, the influence of biomass decay must be considered if long time periods are required for substrate utilization (Montgomery, 1984; Andrews, 1984; Wang, 1988).

Sensitivity analyses (Bard, 1974; Robinson and Tiedje, 1983) were used to design optimal experiments for estimating the kinetic parameters. Sensitivity equations were used to predict (i) whether the parameters may be uniquely estimated, (ii) the relative precision of the estimated parameters, and (iii) the range of the independent variable over which the model is most sensitive to changes in the parameters. Computer simulations of different experimental conditions were used to generate the data sets used in the sensitivity analyses. A computer program utilizing the fourth-fifth order Runge Kutter method was used to simultaneously solve Equations 1 and 2 for S and M.

Inhibitory Effects

The manner in which an inhibitor influences the kinetics of a biological reaction is quite complex and depends on a number of factors. Unfortunately our present understanding of these factors and the mode of action of most inhibitory organic compounds on anaerobic microorganisms is quite limited. Therefore, it is extremely difficult to model the effect of an inhibitor on the kinetic parameters using mechanistic models. The general approach has been to assume empirical models developed for pure enzymes systems to be applicable to biological systems (Lewandowski, 1985; Volskay and Grady, 1988). However, this assumption may not always apply because the effects of inhibitory compounds on living microorganisms are more complex that those on pure enzymes.

Inhibitors are classified as competitive, noncompetitive, uncompetitive, and mixed, depending on how they influence the maximum specific substrate utilization rate (k) and the half saturation coefficient (K_s). In competitive inhibition, the mass action of the substrate overcomes the inhibitory effects so that the observed k is the same as for normal, uninhibited reactions, but the observed K_s increases as toxicant concentration increases. In non-competitive inhibition, K_s does not change as toxicant concentration is increased but k is reduced. In uncompetitive inhibition, both k and K_s are decreased to the same extent. In some cases, a combination of competitive and non-competitive inhibition occurs to produce mixed inhibition. These effects can be represented with simple reversible linear inhibition models (Patterson and Brezonik, 1969) as shown in Table 1, where k_o and K_{so} are the kinetic coefficients in the absence of inhibitory substances, k and K_s are the apparent kinetic coefficients in the presence of inhibitory substances, and k_i, K_i are the inhibition coefficients and I is the inhibitor concentration.

The procedure for determining values of the inhibition coefficients involve first determining values of k and K_s using the non-linear least squares method described above. These values were then entered into the linear form of the inhibition coefficient terms expressed as follows:

$$\frac{1}{k} = \frac{1}{k_o} + \frac{I}{k_i\ k_o} \qquad (13)$$

and

$$K_s = K_{so} + I\left(\frac{K_{so}}{K_i}\right) \qquad (14)$$

These relationships, relative to the classification of inhibition effect, are illustrated in Figure 4.

EXPERIMENTAL PROGRAM

Experimental Design

The experimental program consisted of two phases. Batch screening tests (Young and Tabak, 1989), were used in Phase I to select the concentration levels of toxicants -- representing low, intermediate and moderately high toxicity -- to be used for kinetics testing. Batch tests conducted in Phase II were designed to assess the effect of the toxicant on the rate of acetate degradation at different toxicant levels and initial acetate concentrations. The procedure was based on the batch Anaerobic Toxicity Assay (ATA) described by Owen, et al. (1976), but as modified by Johnson and Young (1983). Batch bioassay techniques were employed for testing purposes because they are relatively simple and tests can be performed within a relatively short period of time, thereby preventing possible major shifts in the microbial population within the test reactor. Also, the effects of different environmental conditions can be studied within a reasonable period of time resulting in a test program that is time, labor, and cost effective.

Anaerobic seed cultures were grown in 12-L laboratory acetate-enriched Master Culture reactors maintained at 35°C and operated at a 20-day solids retention time (0.05 day^{-1} dilution rate). The Master Culture Reactors were fed one time per day on a draw-and-fill basis at an organic loading rate of 1.0 g COD/L-day using acetate as the base organic substrate. Nutrient/Mineral/Buffer medium described by Young and Tabak, 1989, was used in the Master Culture Reactors as well as all cultures transferred to serum bottles. Unacclimated cultures were used to minimize the biodegradation of the toxic organic chemicals throughout the duration of the test.

Six compounds representing two homologous series of chloro-substituted phenols and chloro-substituted anilines were selected for testing from a list of organic chemicals identified by the U. S. Environmental Protection Agency (EPA) as candidates for toxicity assessment (Young and Tabak, 1989) (Table 2). These compounds were selected because they are commonly encountered in industrial waste streams and also because of the overall concerns regarding their toxic effects on the environment.

The experimental setup is shown in Table 3. The purpose of the Test Control unit (without toxicant) was to provide a measure of the "baseline" kinetic parameters which would serve as a basis for comparison. An abiotic Control was included to determine if acetate conversion was affected by non-biological reactions (such as adsorption and volatilization) that must be accounted for in the test measurements. This was accomplished by inhibiting the growth of the seed culture with mercuric chloride preservative solution to give 100 mg $HgCl_2$/L. The test duplicate was used to assess variability between the test replicates.

The procedure involved transferring 400 mL of seed culture, under anaerobic conditions, to each of a number of 500 mL serum bottles which had previously been flushed with a mixture of 70% nitrogen and 30% carbon dioxide. Prior to the start of the test, the cultures were stabilized over a period of three to four days by daily removing 20 mL of mixed liquor from each serum bottle and adding 20 mL of stock acetate substrate (20 g COD/L). Total daily gas production was monitored manually with a syringe or by means of an automatic anaerobic respirometer to verify activity of the transferred cultures. The test cultures were selected from among the serum bottles that consistently produced the expected amount of gas according to the stochiometry of the reaction.

The tests were begun after applying a slug dose of the toxicant to the test units and test duplicate at carefully selected concentration levels determined from Phase I along with acetate at a desired initial concentration (see Table 2). Ten millimeter samples were removed from each serum bottle at close time intervals for at least 24 hours, transferred to sealed, septum-capped vials containing two drops of 8 g/L mercuric chloride for sample preservation and centrifuged (G = 5125) for 30 minutes. Five milliliters of centrate were then transferred to clean 5 mL septum-capped vials containing one drop of concentrated sulfuric acid (to adjust pH to < 2) prior to gas chromatographic analysis. Samples were analyzed within the shortest possible time after collection (typically within 48 hours) to minimize losses due to sample storage.

Analytical Procedures

The samples were analyzed for residual acetate by gas chromatography using a glass column (1.8 m x 2.0 mm ID) packed with 10% SP-1200/1% H_3PO_4 on 80/100-mesh Chromosorb W A W and operated isothermally at 125°C. Phosphoric acid-treated wool was used to retain the packing in the column and to prevent peak tailing. The injection port was operated at 200°C and contained a glass liner that was changed frequently to prevent the build up of organic char. The flame ionization detector was heated to 250°C. Nitrogen at a flow rate of 40 mL/min was used as the carrier gas.

The samples were also analyzed for soluble residual toxicants to ascertain whether any significant degradation or loss of the toxicant occurred over the duration of the test. The chloro-substituted phenols were analyzed using a glass column (2 m x 2 mm ID) packed with 1% SP-1240 DA on 100/120 Supelcoport. The column was temperature programmed from 70°C to 200°C at 8°/min and the flame ionization detector was maintained at 250°C. Nitrogen (30 mL/min) was the carrier gas. Direct aqueous injection was used for all compounds, and quantitative analyses were based on absolute response factors. No analysis was made for the chloroanilines.

RESULTS AND DISCUSSION

Phase I Results

The results of Phase I prescreening tests are presented in Figure 5 for the six toxicants studied. The graphs show gas production for each test reactor expressed as a percentage of the cumulative gas produced by the control at the end of the incubation period. Correction for gas produced in the seed blank were applied to all test reactors.

Chlorophenols

The chlorophenols exhibited distinctly different toxicity effects on gas production (Figures 5A, 5B and 5C). Figure 5A shows that 2-chlorophenol was only slightly toxic at the 100 mg/L toxicant level but showed gradually increasing inhibitory effects as the concentration was increased over the 100 to 1000 mg/L toxicant range. Gas production was essentially completely inhibited at 1000 mg/L, with 50 percent inhibition of gas production occurring at about the 600 mg/L toxicant level. These results indicate no evidence of recovery throughout the 24 hour test period. Based on these results, 300 and 600 mg/L 2-chlorophenol concentrations were selected for Phase II tests. The toxicity of 3-chlorophenol increased steadily over the 100 to 300 mg/L toxicant levels, but exhibited a dramatic increase somewhere between 300 and 600 mg/L levels (Figure 5B). At 600 mg/L and above, 3-chlorophenol was highly toxic and caused gas production to essentially cease. From these results, 100 and 300 mg/L 3-chlorophenol concentrations were selected for Phase II tests. Inhibition of gas production by 4-chlorophenol increased at concentrations up to 300 mg/L with some evidence of recovery at the 300 mg/L level as indicated by the lag period of about 5 hrs. At 600 mg/L, gas production was totally inhibited. Consequently, 50, 100, 300 mg/L 4-chlorophenol concentrations were selected for testing in Phase II. In all cases, test duplicates show very close and consistent agreement as demonstrated by the almost identical gas production curves.

Chloroanilines

The chloroanilines exhibited patterns of toxic effects on gas production similar to those caused by the chlorophenols except at relatively higher toxicant concentrations. Only a modest increase in toxicity occurred at 2-chloroaniline concentrations up to 800 mg/L, while 3-chloroaniline showed relative gas production of 70 percent up to the 1600 mg/L level. This indicates that 2- and 3-chloroaniline were relatively less toxic than 4-chloroaniline. Based on these results, 200, 400 and 800 mg/L toxicant concentrations were selected for these two compounds for Phase II tests. Inhibition of gas production by 4-chloroaniline showed a pattern of recovery at the 200 - 800 mg/L toxicant concentration range so that 100, 200 and 300 mg/L concentrations were selected for Phase II tests.

Phase II Results

Phase II tests were conducted to assess the impact of the six toxic organic chemicals on the kinetics of the acetoclastic methanogenic reaction (see Table 2). The initial substrate concentrations, which ranged from 375 to 938 mg/L acetate, were chosen to provide some overlap of the substrate uptake curves obtained with each level of initial substrate concentration. The results of these tests are shown graphically on the left side of Figures 6 through 11 along with the computer-simulated substrate and biomass concentrations curves representing the best-fit of the measured data. The kinetic parameters k and K_s were estimated from experimental data obtained under each test condition (Tables 4 and 5).

Effect of Initial Substrate Concentration

The variation in the values of the kinetic coefficients between individual tests is due in part to the sensitive nature of k and K_s in different regions of the substrate uptake curve. Sensitivity analyses indicate that k is most sensitive to the accuracy and volume of data in the zero-order region, while K_s

is most sensitive in the mixed-order region. However, sensitivity analyses do not account for the effects of the inhibitor on substrate utilization, and as such the regions of the substrate uptake curve where the kinetic coefficients are most sensitive could not be precisely determined in advance. Also the volume of culture typically used in the batch bioassay tests limited the quantity and frequency of samples that could be withdrawn for analysis. Hence the distribution of the experimentally measured points along the substrate uptake curve could significantly affect the accuracy of the estimated kinetic coefficients.

The data presented in Figures 6 through 11 and the results summarized in Tables 4 and 5 clearly illustrate one of the problems associated with using single batch uptake curves for estimating kinetic coefficients: that is the validity of the kinetic coefficients obtained with data from a single substrate uptake curve may be questionable because such data may not always contain sufficient points to span the sensitive regions of the curve at reasonably close time intervals. To address this problem, the authors adopted the approach of combining the data obtained from tests conducted at various levels of initial substrate concentrations for each level of toxicant concentration.

The procedure used to combine the data points was as follows: a calculated curve was fitted to the data obtained at the highest level of initial acetate concentration. The time required to degrade the acetate to the next level of initial acetate concentration was determined from the fitted curve to the nearest 0.6 minutes of reaction time. The time value associated with this point at the higher concentration curve was then added to the actual measured time values for the data obtained at the lower concentration. The time shift allowed the two sets of data to be treated as if it were produced from the same reaction. This procedure was repeated for the other levels of initial substrate concentration. In view of the sensitivity of the kinetic coefficients to S_o, the initial added acetate concentration was used in the model instead of the initial measured quantities. The graph segments on the right side of Figures 6 through 11, illustrate the effect of combining the substrate uptake curves obtained for different initial acetate concentrations into a single curve. As evident from these graphs, this procedure improved the distribution of the data points significantly, thereby providing a more realistic data set for the estimation of the kinetic parameters.

Generally k and K_s values for the combined data fall within the range of values calculated using data from individual tests (Tables 4 and 5).

Effect of Toxicant Concentration

Figure 12 illustrates the effect of toxicant concentration on acetate utilization for the six toxicants tested. These plots represent the substrate uptake reactions calculated using the combined data at the different levels of initial substrate concentrations as discussed above. In each case, as the toxicant concentration was increased, the rate of acetate utilization decreased. The comparison of the kinetic coefficients revealed significant changes in both the maximum rate of acetate conversion, k, and the half- velocity coefficient, K_s, for both chlorophenols and chloroanilines. The relative effect of the toxicant was manifested in a decrease in the value of k and/or an increase in the value of K_s for the combined data (Tables 4 and 5).

Effect of Inhibition Type

When the calculated kinetic coefficients were substituted into the linear reversible models represented by Equations 11 and 12 the results are shown graphically in Figure 13 and the resulting inhibition coefficients, k_i and K_i, are listed in Table 6. The data for both the chlorophenols and chloroanilines appear to conform best to the mixed inhibition model with k decreasing and K_s increasing as the toxicant concentration increased. However, the changes in K_s are significantly greater than those in k, indicating the predominance of competitive inhibition mechanisms. Figures 13B and 13D illustrate this effect with the $1/k$ curves being almost identical. However, a slight deviation from this trend was observed for 4-chlorophenol.

DISCUSSION

The main benefits of categorizing inhibitory effects lies in the ability to use the data to predict inhibitory effects based on chemical classification and functionality. Hartmann and Laubenberger (1969) showed trends in competitive versus non-competitive inhibitory effects for metal ions generally associated with ionic charge and molecular weights. Major potential benefits will arise if similar, yet more comprehensive, relationships can be derived between organic chemical structure and inhibitory activity. In the present study, the toxic effects were clearly related to the molecular structure of the toxicants in each homologous series. A direct correlation was observed between the degree of toxicity and the position of the substituted radical on the benzene ring. For the chlorophenol series the results showed that the toxicity increased in relation to the position of the substituted radical on the benzene ring with 4-chlorophenol > 3-chlorophenol > 2-chlorophenol and 4-chloroaniline > 3-chloroaniline > 2-chloroaniline. However, the chlorophenols exhibited a relatively greater degree of toxicity per unit concentration than did the chloroanilines which confirms the results of Phase I.

The values of k_o and K_{so} were remarkably constant for combined data for the six control tests (see Tables 4 and 5) with a coefficient of variability of 0.87% for k_o and 8.7% for K_{so} (Table 6). The variation in coefficients was much greater for the tests at individual initial acetate concentrations. In this case, the coefficients of variability were 6.8% for k_o and 39.3% for K_{so}. This illustrates the problems with using individual tests to provide accurate measures of kinetic coefficients.

The kinetic coefficients measured through the process described above are thought to be truly intrinsic, that is, unaffected by outside factors such as method of system operation, or the presence of extraneous materials such as inactive solids or other toxicants. The method of using active growth as the biomass term in the substrate uptake and biological growth equations is felt to correct for differences that might arise if measurements are made at different solids retention times.

CONCLUSIONS

Based on the results of tests described in this paper, the following conclusions could be drawn:

1. Bench-scale tests were used successfully for assessing the impact of six compounds -- representing two homologous series of chlorosubstituted phenols and chlorosubstituted anilines -- on the kinetics of acetoclastic methanogenesis.

2. A mathematical model based on a finite difference non-linear least-squares algorithm was developed to estimate the kinetic coefficients k and K_s using a two-dimensional grid search technique.

3. Different levels of toxicant concentrations caused significant changes in the kinetic coefficients k and K_s with the chlorophenols showing relatively greater toxic effects. Initial acetate concentrations had no effect on the values of the baseline substrate uptake and biomass growth coefficients.

4. Both chlorophenols and chloroanilines exhibited mixed inhibition with a shift from predominantly non-competitive inhibition with the 2-chloro isomers to predominantly competitive inhibition with the 3- and 4-chloro isomers.

5. Structure-activity relationships for both series of chlorophenols and chloroanilines show that the relative toxicity increased as the position of the substituted chlorine radical changed from the ortho- to the meta- to the para- positions on the benzene ring.

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Table 1. Effects of inhibitors on kinetic parameters based on simple reversible linear inhibition models

Inhibition Type	Effect on k	Effect on K_s
Competitive	$k = k_o$	$K_s = K_{so}(1 + I/K_i)$
Non-competitive	$k = k_o/(1 + I/k_i)$	$K_s = K_{so}$
Uncompetitive	$k = k_o/(1 + I/k_i)$	$K_s = K_{so}/(1 + (I/K_i))$
Mixed	$k = k_o/(1 + I/k_i)$	$K_s = K_{so}(1 + (I/K_i))$

Table 2. Experimental design of phase II kinetic tests

Run Series	Toxicant	Toxicant Concentrations (mg/L)	Initial Acetate Concentration (mg COD/L)
1	2-Chlorophenol	0,300,600	500,1000
2	3-Chlorophenol	0,100,300	500,1000
3	4-Chlorophenol	0,50,100,300	500,1000
4	2-Chloroaniline	0,200,400,800	400,700,1000
5	3-Chloroaniline	0,200,400,800	400,700,1000
6	4-Chloroaniline	0,100,200,300	500,1000

Table 3. Experimental setup for phase II kinetic tests

No. of Bottles	Materials Added	Function
1	Seed + acetate	Test Control
1	Seed + acetate + $HgCl_2$	Abiotic Control
3	Seed + acetate + toxicant at 3 dose levels	Test units
1	Seed + acetate + duplicate toxicant dose	Test duplicate

Table 4. The effect of initial substrate and chlorophenol concentrations on the value of the estimated kinetic coefficients k and K_s.

2-CHLOROPHENOL						
	So=938 mg/L HAC		So=469 mg/L HAC		Combined Data	
Tox. Conc (mg/L)	k (hr-1)	Ks (mg/L)	k (hr-1)	Ks (mg/L)	k (hr-1)	Ks (mg/L)
0	0.2395	45.0	0.2618	51.4	0.2390	40.8
300	0.1323	38.2	0.1369	48.8	0.1347	50.6
600	0.0908	58.2	0.0991	68.1	0.0911	56.6

3-CHLOROPHENOL						
	So=938 mg/L HAC		So=469 mg/L HAC		Combined Data	
Tox. Conc (mg/L)	k (hr-1)	Ks (mg/L)	k (hr-1)	Ks (mg/L)	k (hr-1)	Ks (mg/L)
0	0.2402	41.0	0.2614	53.1	0.2398	39.2
100	0.1725	52.6	0.1661	46.4	0.1738	59.8
300	0.1319	94.1	0.1331	92.1	0.1314	90.8

4-CHLOROPHENOL								
	So=938 mg/L HAC		So=656 mg/L HAC		So=375 mg/L HAC		Combined Data	
Tox. Conc (mg/L)	k (hr-1)	Ks (mg/L)	k (hr-1)	Ks (mg/L)	k (hr-1)	Ks (mg/L)	k (hr-1)	Ks (mg/L)
0	0.2384	38.2	0.2437	45.0	0.2711	53.1	0.2391	39.5
50	0.2268	60.0	0.2345	61.5	0.2361	59.9	0.2276	60.3
100	0.2032	93.7	0.2094	102.6	0.2155	109.5	0.2044	97.7
300	0.1642	156.3	0.1725	177.2	0.1766	184.5	0.1662	166.8

Table 5. The effect of initial substrate and chloroaniline concentrations on the values of the estimated kinetic coefficients k and K_s.

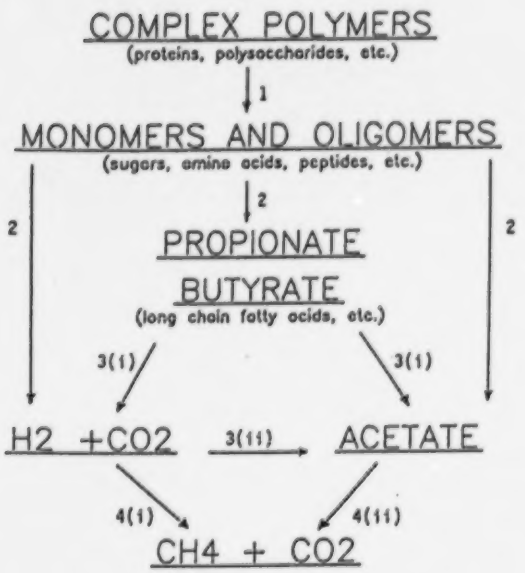
2-CHLOROANILINE								
	So=938 mg/L HAC		So=656 mg/L HAC		So=375 mg/L HAC		Combined Data	
Tox. Conc (mg/L)	k (hr-1)	Ks (mg/L)	k (hr-1)	Ks (mg/L)	k (hr-1)	Ks (mg/L)	k (hr-1)	Ks (mg/L)
0	0.2370	38.3	0.2968	113.2	0.2615	55.1	0.2401	45.2
200	0.1968	72.8	0.1605	16.5	0.2285	83.4	0.1878	54.7
400	0.1462	86.0	0.1396	61.3	0.1353	58.1	0.1437	77.9
800	0.1035	91.2	0.1094	103.8	0.1095	98.7	0.1045	95.0

3-CHLOROANILINE								
	So=938 mg/L HAC		So=656 mg/L HAC		So=375 mg/L HAC		Combined Data	
Tox. Conc (mg/L)	k (hr-1)	Ks (mg/L)	k (hr-1)	Ks (mg/L)	k (hr-1)	Ks (mg/L)	k (hr-1)	Ks (mg/L)
0	0.2302	27.8	0.2674	79.0	0.2505	37.8	0.2361	40.8
200	0.1931	71.6	0.1806	45.1	0.1754	24.6	0.1863	53.5
400	0.1526	106.4	0.1300	51.9	0.1208	49.1	0.1480	101.1
800	0.1073	168.5	0.1027	152.8	0.1009	135.8	0.1043	154.0

4-CHLOROANILINE						
	So=938 mg/L HAC		So=469 mg/L HAC		Combined Data	
Tox. Conc (mg/L)	k (hr-1)	Ks (mg/L)	k (hr-1)	Ks (mg/L)	k (hr-1)	Ks (mg/L)
0	0.2361	40.1	0.2626	47.2	0.2345	33.5
100	0.2160	64.0	0.2020	36.8	0.2120	56.4
200	0.1874	75.1	0.1831	57.3	0.1847	67.4
300	0.1573	121.5	0.1418	74.7	0.1533	106.8

Table 6. Summary of kinetic and inhibition coefficients

TOXICANT	KINETIC COEFFICIENTS				INHIBITION COEFFICIENTS	
	k, day		K_s, mg/L			
	Ind.	Comb.	Ind.	Comb.	k_i	K_i
2-CHLOROPHENOL	0.2507	0.2390	48.2	40.8	365	157
3-CHLOROPHENOL	0.2508	0.2398	46.6	39.2	391	240
4-CHLOROPHENOL	0.2511	0.2391	45.4	39.5	671	104
- - - - - - - -						
2-CHLOROANILINE	0.2651	0.2401	68.9	45.2	600	705
3-CHLOROANILINE	0.2494	0.2361	48.2	40.8	610	233
4-CHLOROANILINE	0.2494	0.2345	43.7	33.5	550	136
$\bar{X}$, mg/L =	0.2527	0.2381	50.2	39.8		
Sd, mg/L =	0.0061	0.0023	9.3	3.8		
Cv, % =	2.4	0.95	18.6	9.5		
Ind $\bar{X}$, mg/L =	0.2532		51.0			
Ind Sd, mg/L =	0.0179		20.7			
Ind Cv, % =	7.1		40.6			



1: HYDROLYSIS
2: FERMENTATION
3: (i) SYNTROPHIC ACETOGENESIS
(ii)HOMOACETOGENESIS
4: (i) HYDROGENOTROPHIC METHANOGENESIS
(ii)ACETOCLASTIC METHANOGENESIS

Figure 1. Schematic diagram showing the four major phases of anaerobic biotransformation of organic materials (from Wilkie and Colleran, 1986).

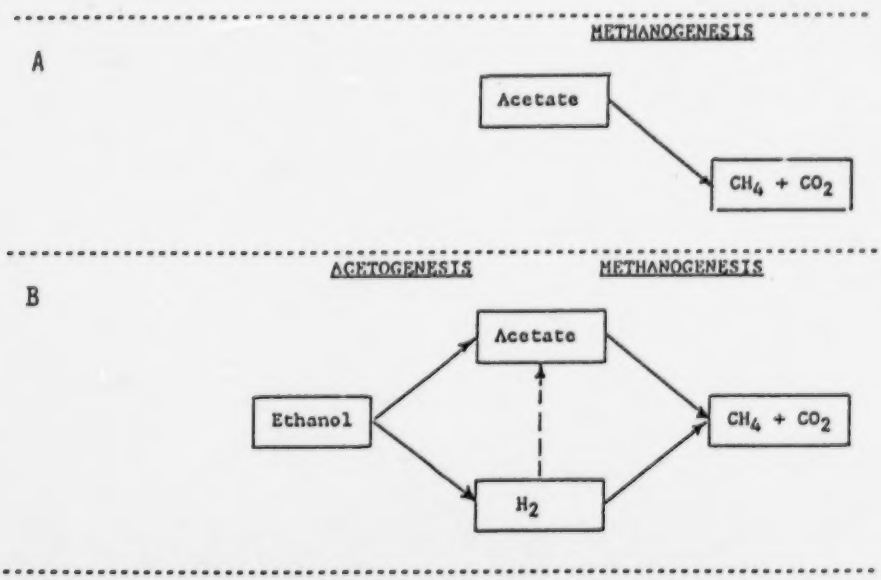


Figure 2. Schematic representations of two reaction levels during anaerobic transformations of organic compounds.

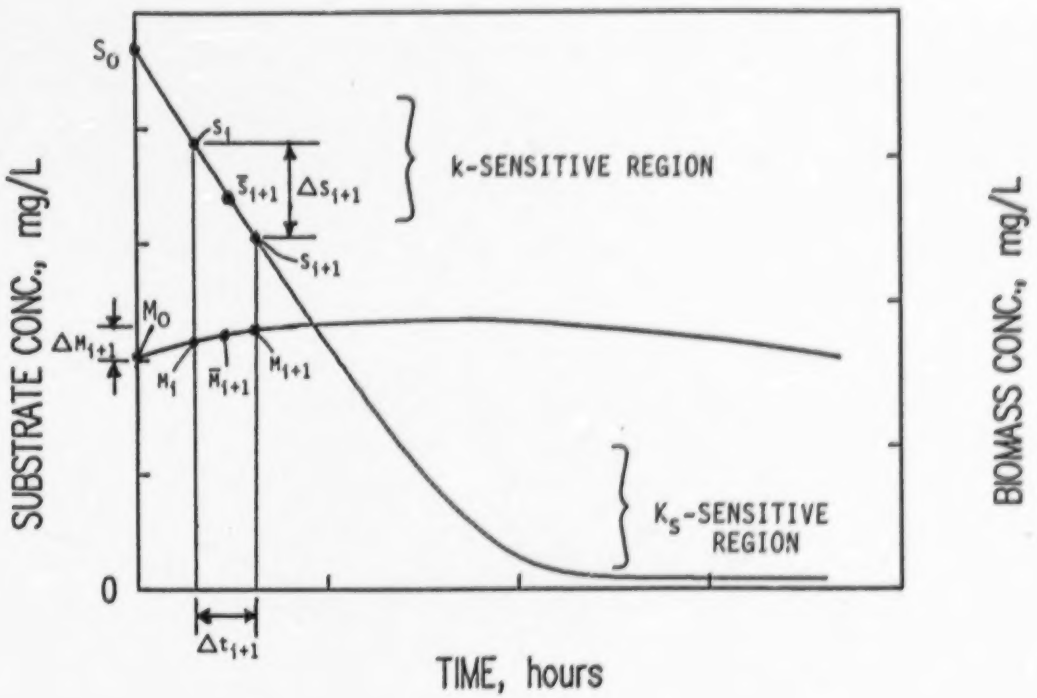


Figure 3. Graphical illustration of the progress of the substrate utilization and biomass curves for the Monod equations based on a finite difference approach.

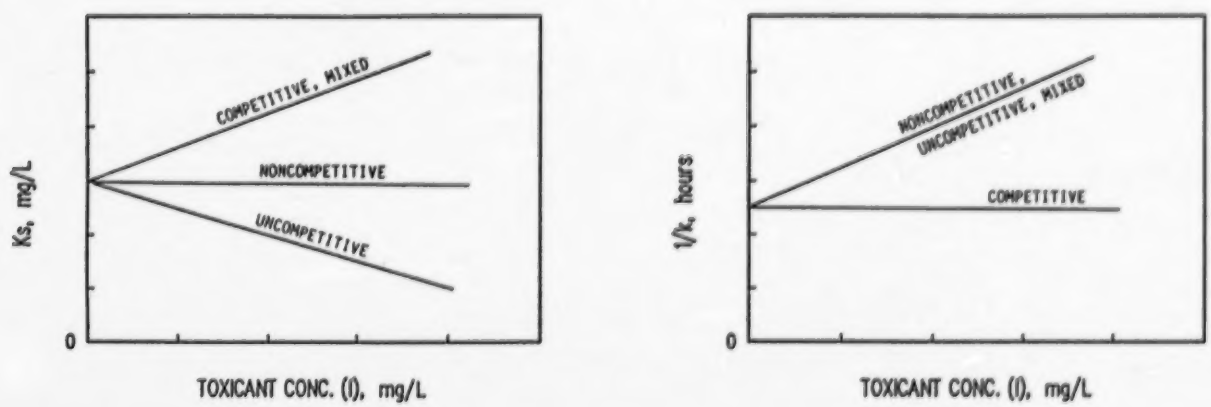


Figure 4. Graphical illustration of changes in k and K_s for acetate conversion with increases in toxicant concentration.

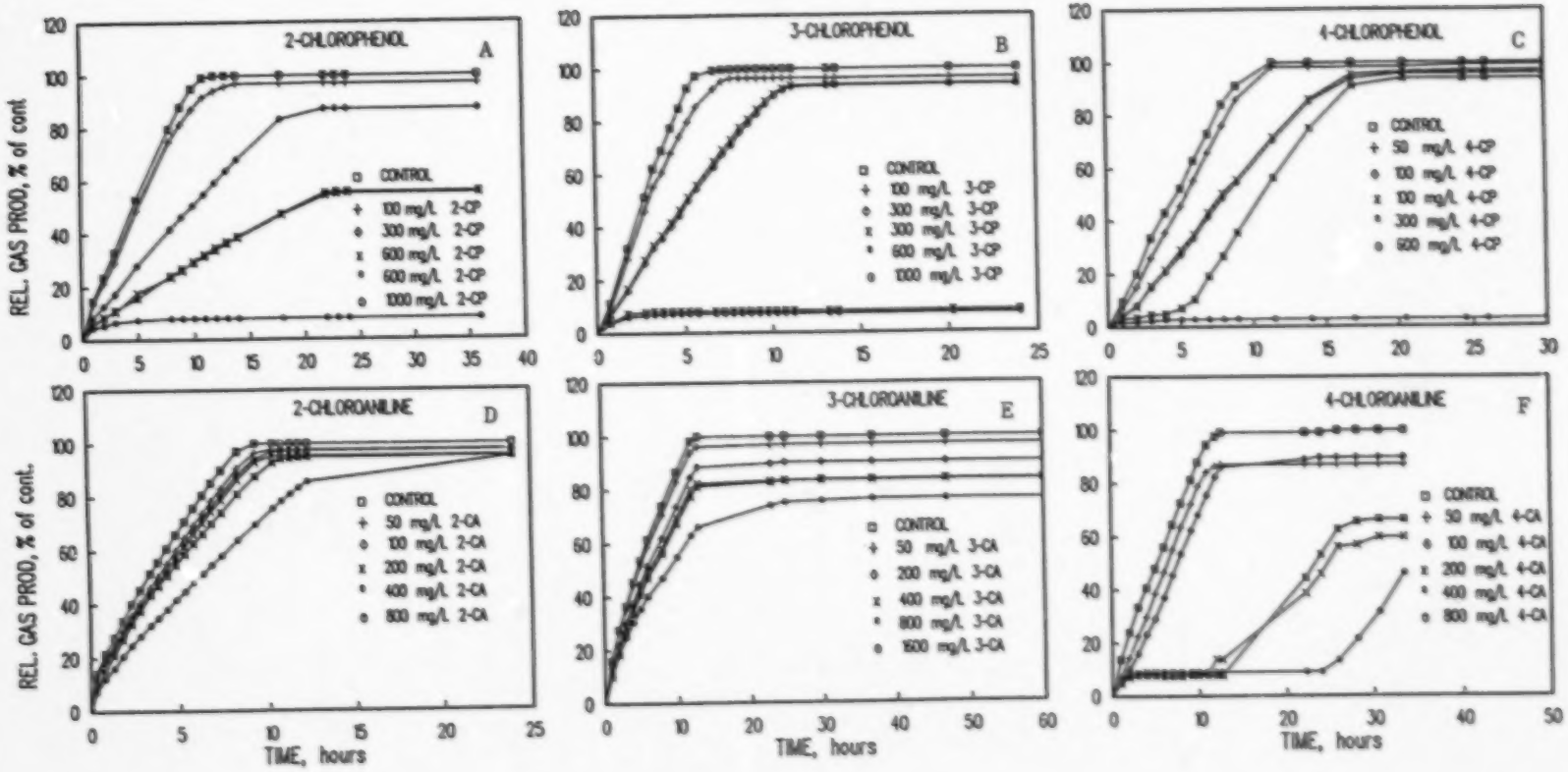


Figure 5. Relative gas production measured during Phase I.

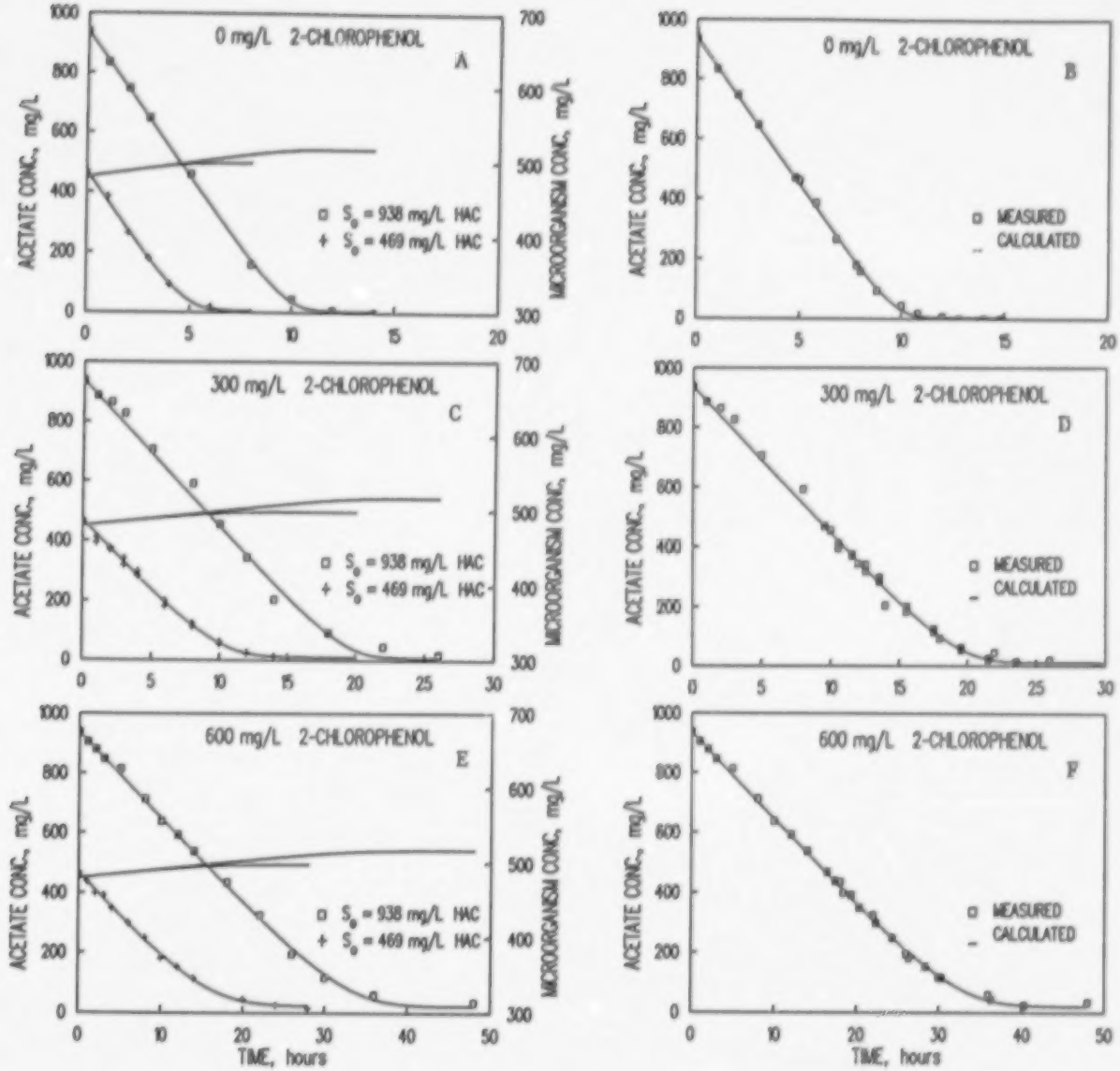


Figure 6. Kinetic test results showing separate acetate utilization and biomass curves at initial substrate concentrations of 938 and 469 mg/L acetate, and 300 and 600 mg/L 2-chlorophenol levels (left segments). Segments on the right show the corresponding composite curves.

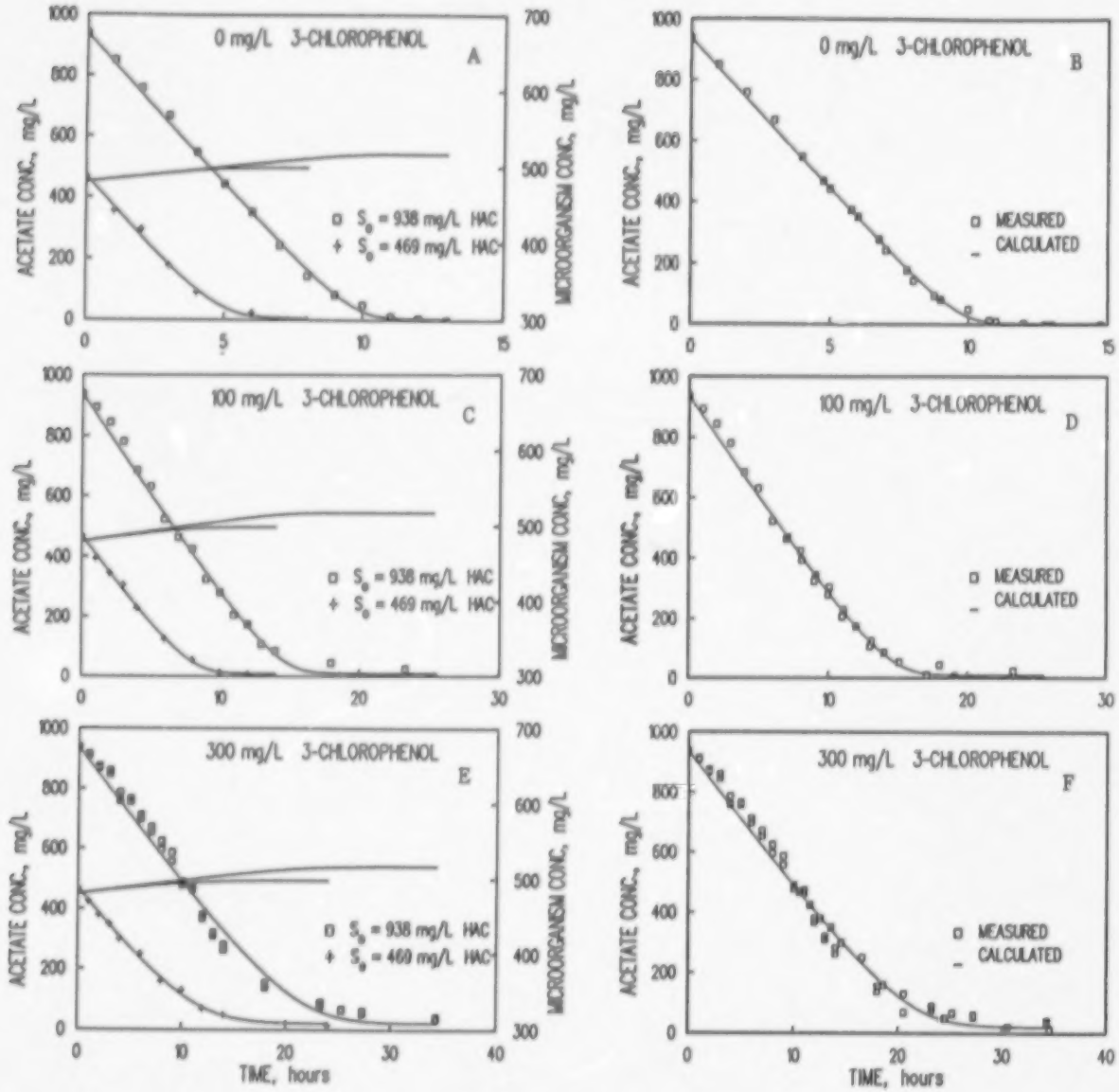


Figure 7. Kinetic test results showing separate acetate utilization and biomass curves at initial substrate concentrations of 938 and 469 mg/L acetate and 100 and 300 mg/L 3-chlorophenol levels (left segments). Segments on the right show the corresponding composite curves.

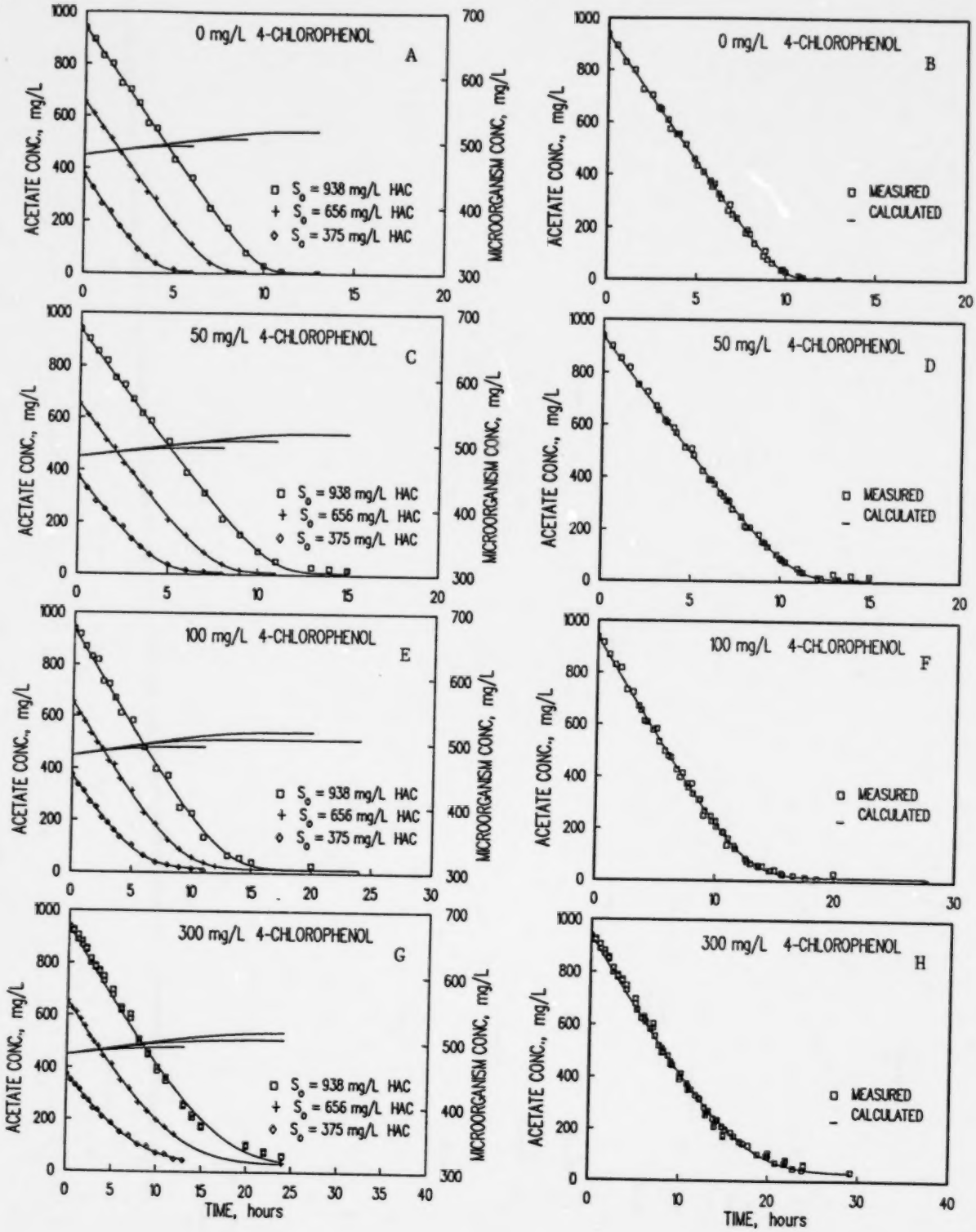


Figure 8. Kinetic test results showing separate acetate utilization and biomass curves at initial substrate concentrations of 938, 656, and 375 mg/L acetate, and 50, 100, and 300 mg/L 4-chlorophenol levels (left segments). Segments on the right show the corresponding composite curves.

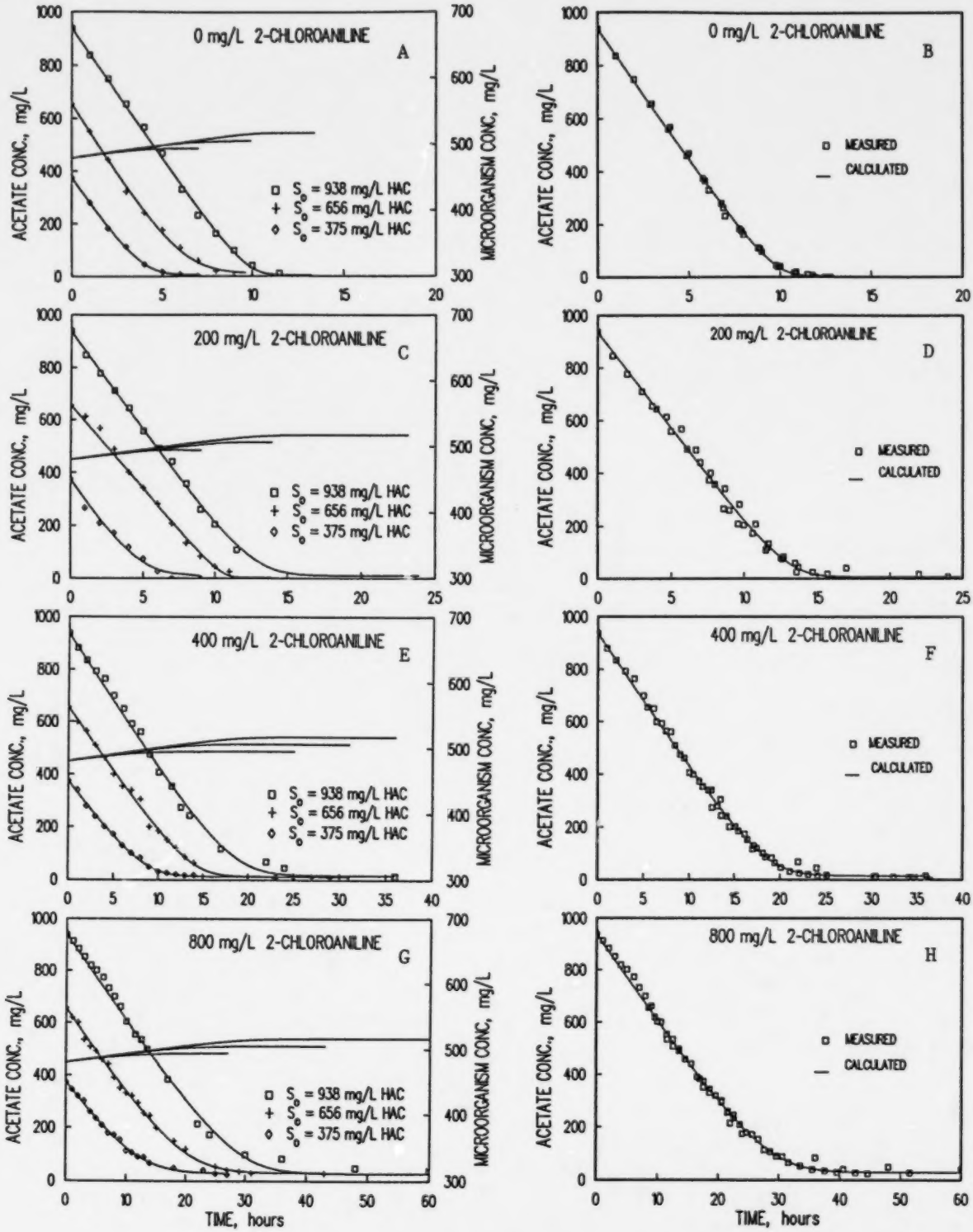


Figure 9. Kinetic test results showing separate acetate utilization and biomass curves at initial substrate concentrations of 938, 656, and 375 mg/L acetate, and 200, 400, and 800 mg/L 2-chloroaniline levels (left segments). Segments on the right show the corresponding composite curves.

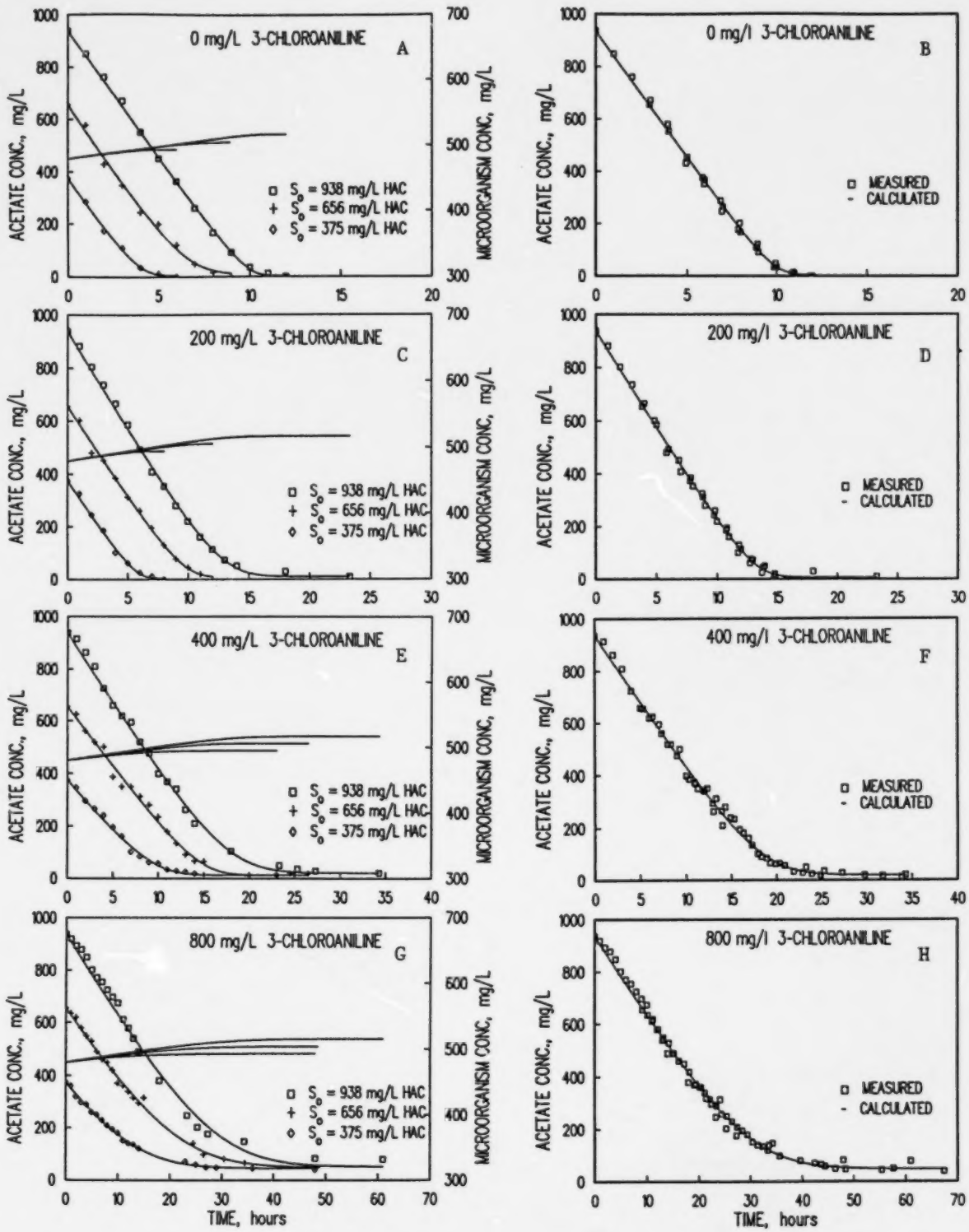


Figure 10. Kinetic test results showing separate acetate utilization and biomass curves at initial substrate concentrations of 938, 656, and 375 mg/L acetate, and 200, 400, and 800 mg/L 3-chloroaniline levels (left segments). Segments on the right show the corresponding composite curves

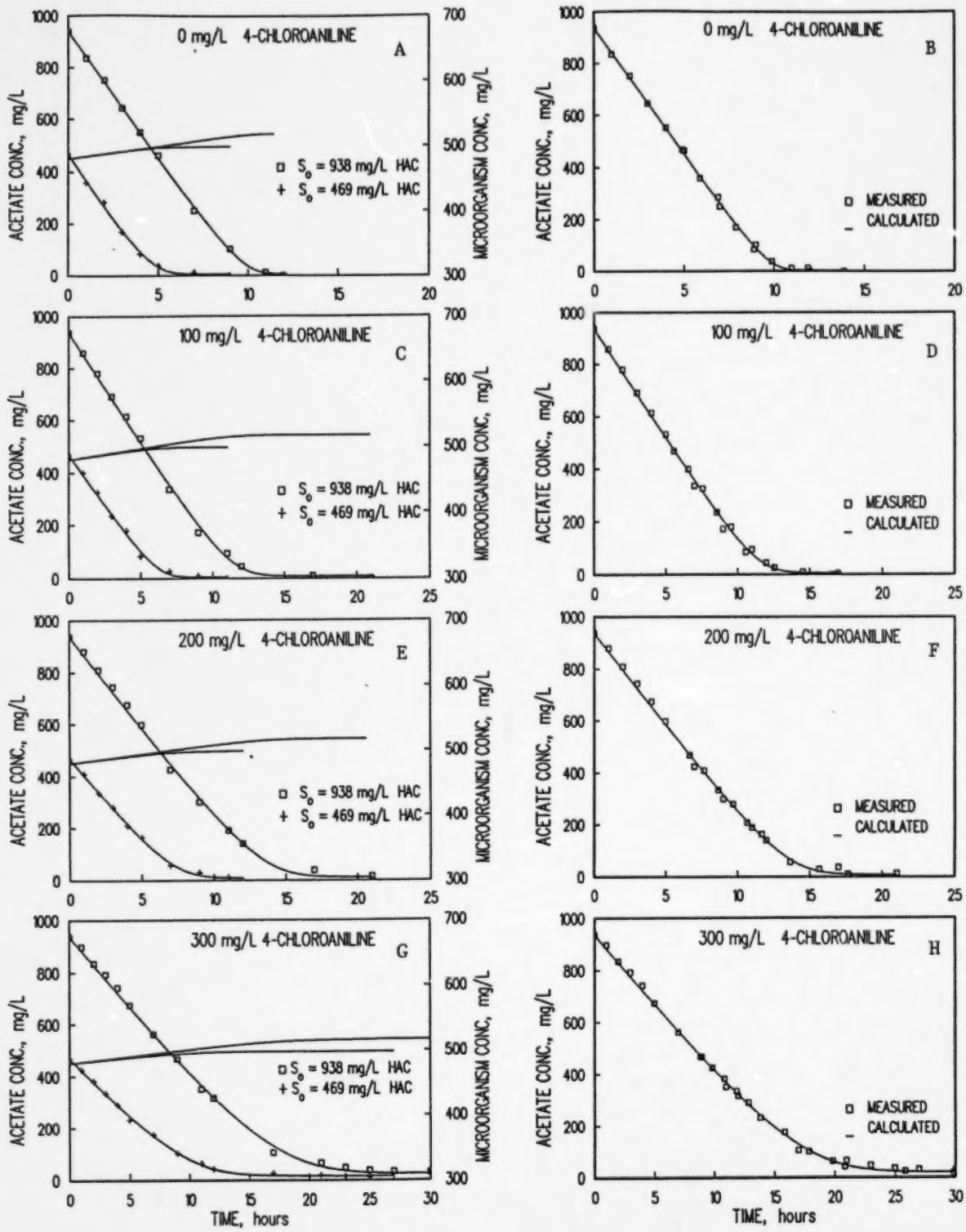


Figure 11. Kinetic test results showing separate acetate utilization and biomass curves at initial substrate concentrations of 938 and 469 mg/L acetate, and 100, 200, and 300 mg/L 4-chloroaniline levels (left segment). Segments on the right show the corresponding composite curves.

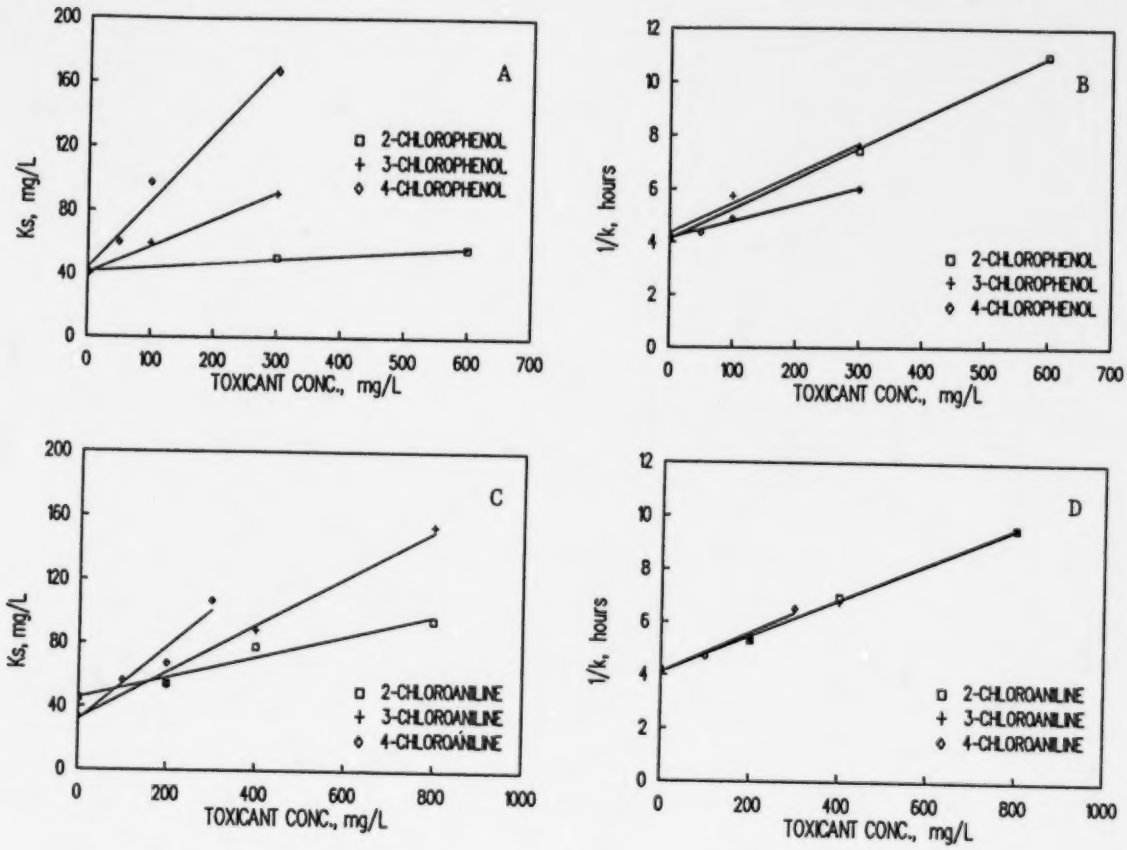


Figure 13. The effect of toxicant concentration on k and K_s for chlorophenol (upper segments), and chloroaniline (lower segments).

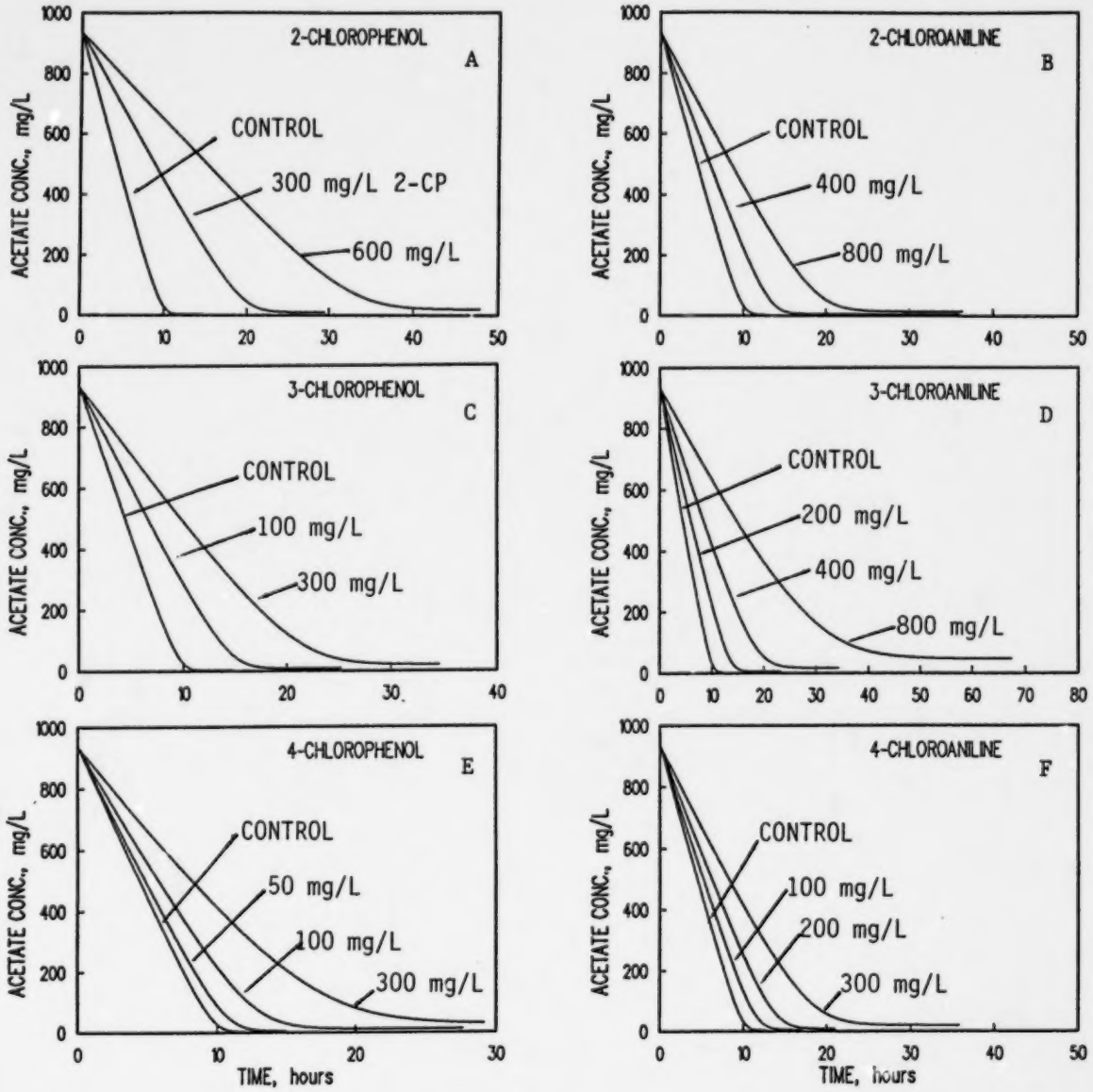


Figure 12. Kinetic test results showing the effect of various levels of chlorophenol concentrations (left segments), and chloroaniline concentrations (right segments).